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(57) Abstract

MAGE-3 tumor antigens are useful for overcoming tolerance to tumors.

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BABOON MAGE-3 HOMOLOGS, DNA ENCODING THE HOMOLOGS, AND A PROCESS FOR THEIR USE

Background of the Invention

One of the major research goals in cancer therapy is to trigger the patient's immune system to actively respond to proliferation of a tumor. Certain pathologies appear to be resistant to the immune system because they exhibit characteristics that result in tolerance by the host, or they disable the capability of the host's immune system to combat them. The present invention is designed to overcome the tolerance to tumor antigens. See Dalgleish, Eur. Jour. Cancer, 30A:1029-1035 (1994); Scientific American, September 1994, page 102; and Finn, Clin. Immunol. and Immunopath., 71:260-262 (1994) which are hereby incorporated by reference in their entireties.

Summary

The present invention is a nucleic acid sequence which codes for a baboon MAGE-3 homolog and hybridizes to any of sequences A, B, C, D, or F from Table 1 under stringent conditions. The invention also includes expression vectors, control sequences, and host cells to express the polypeptide encoded by the nucleic acid sequence. The use of the nucleic acid sequence in gene therapy is also claimed including gene therapy designed to elicit immune responses. The present invention also includes claims to the polypeptide sequence coded by the nucleic acid sequences, immunologically active polypeptide fragments, and formulations of the polypeptide. It is envisioned that the polypeptide will be used to stimulate the immune system of humans. Preferably an adjuvant will be added to the formulation to stimulate the human immune system.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, immunology, and vaccine development that are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, et al.,

MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989); DNA CLONING, VOLUMES I AND II (D.N Glover ed. 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed, 1984); NUCLEIC ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds. 1984); TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins eds. 1984); ANIMAL CELL CULTURE (R.I. Freshney ed. 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984); the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory), Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively), Mayer and Walker, eds. (1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London), Scopes, (1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Second Edition (Springer-Verlag, N.Y.), HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, VOLUMES I-IV (D.M. Weir and C. C. Blackwell eds 1986); and VACCINES (R.W. Ellis, ed., 1992, Butterworth-Heinemann, London).

Standard abbreviations for nucleotides and amino acids are used in this specification. All publications, patents, and patent applications cited herein are incorporated by reference.

Definitions

"Homology" refers to the degree of similarity between x and y. The correspondence between the sequence from one form to another can be determined by techniques known in the art. For example, they can be determined by a direct comparison of the sequence information of the polynucleotide. Alternatively, homology can be determined by hybridization of the polynucleotides under conditions which form stable duplexes between homologous regions (for example, those which would be used prior to S_1 digestion), followed by digestion with single-stranded specific nuclease(s), followed by size determination of the digested fragments.

"Hybridization" refers to the association of two nucleic acid sequences to one another by hydrogen bonding. Typically, one sequence will be fixed to a solid support

and the other will be free in solution. Then, the two sequences will be placed in contact with one another under conditions that favor their binding. Factors that affect this binding include: the type and volume of solvent; reaction temperature; time of hybridization; agitation; agents to block the non-specific attachment of the liquid phase sequence to the solid support (Denhardt's reagent or BLOTTO); concentration of the sequences; use of compounds to increase the rate of association of sequences (dextran sulfate or polyethylene glycol); and the stringency of the washing conditions following hybridization. See Sambrook, et al., MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989), Volume 2, chapter 9, pages 9.47 to 9.57.

"Stringency" means that the washing conditions in the hybridization reaction should favor association of very similar sequences over sequences that differ. For example, the combination of temperature and salt concentration should be chosen that is approximately 12° to 20°C below the calculated T_m of the hybrid under study. The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the sequence of interest and then washed under conditions of different stringencies. See Sambrook, et al., above at page 9.50.

"Cross priming" means the use of a protein homolog to elicit an immune response in a different species than that from which the protein was derived, such as, the use of a baboon MAGE-3 homolog in a human. For example, the present baboon MAGE homologs are likely to contain many HLA class I, class II, and antibody epitopes of human MAGE-3. Baboon MAGE-3 homologs are also likely to contain a number of non-human/foreign epitopes expected to elicit a vigorous immune response in humans. This vigorous response to foreign epitopes will provide immunological "help" for response to conserved human epitopes via "cross priming".

A "vector" or "plasmid" is a nucleic acid sequence in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment.

"PCR" refers to the technique of polymerase chain reaction as described in Saiki, et al., Nature 324:163 (1986); and Scharf et al., Science (1986) 233:1076-1078; and U.S. Pat. Nos. 4,683,195; and U.S. 4,683,202.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence so that expression of the coding sequence is achieved under conditions compatible with the control sequences.

The term "polynucleotide" or "nucleic acid sequence" as used herein refers to a polymer of nucleotides of any length, preferably deoxyribonucleotides, and is used interchangeably herein with the terms "oligonucleotide" and "oligomer." The term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, as well as antisense polynucleotides. It also includes known types of modifications, for example, the presence of labels which are known in the art, methylation, end "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, replacement with certain types of uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) or charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), introduction of pendant moieties, such as, for example, proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive species, boron, oxidative moieties, etc.), alkylators (e.g., alpha anomeric nucleic acids, etc.).

By "genomic" is meant a collection or library of DNA molecules which correspond to the sequence found in chromosomal DNA as opposed to spliced mRNA. By "cDNA" is meant a DNA sequence that hybridizes to a complimentary strand of mRNA.

As used herein, x is "heterologous" with respect to y if x is not naturally associated with y in the identical manner; i.e., x is not associated with y in nature or x is not associated with y in the same manner as is found in nature.

As used herein, the term "protein" or "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, polypeptides, proteins, and polyproteins, as well as fragments of these, are included within this definition. This term also does not refer to, or exclude, post expression modifications of the protein, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, proteins containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), proteins with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

A polypeptide or protein or amino acid sequence "derived from" or "coded by" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 3-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence.

"Immunogenic" or "immunostimulatory" refers to the ability of a polypeptide to cause a humoral and/or cellular immune response, whether alone or when linked to a carrier, in the presence or absence of an adjuvant.

"Epitope" refers to an antigenic determinant of a peptide, polypeptide, or protein; an epitope can comprise 3 or more amino acids in a spatial conformation unique to the epitope. Generally, an epitope consists of at least 5 such amino acids and, more usually, consists of at least 8-10 such amino acids. Methods of determining spatial conformation of amino acids are known in the art and include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen.

There are different types of "epitopes" recognized by T cell receptors vs. antibodies. A "T-cell epitope" is a contiguous peptide that binds to a class I or II HLA molecule and is presented thereby to a specific T cell receptor; its conformation depends not only on peptide sequence, but on the manner of its binding to the HLA groove. An "antibody epitope" is a part of the surface of a macromolecule that has a particular shape that fits a specific antibody binding site; it may consist of contiguous amino acids, non-contiguous amino acids, and/or other residues such as sugars or haptens.

"Treatment," as used herein, refers to prophylaxis and/or therapy (i.e., the modulation of any disease symptoms). An "individual" indicates an animal that is susceptible to treatment, including but not limited to, primates, including humans. A "vaccine" or a "therapeutic vaccine" is an immunogen, capable of eliciting protection, whether partial or complete, against a tumor or a microbial pathogen. See U. S. Pat. Nos. 5,141,742, 4,720,386, 5,194,384, and 4,877,611 which are hereby incorporated by reference in their entireties. The tumors which can be treated by the present invention include all tumor types that present MAGE-3 or similar proteins on their surface. Preferably, the tumor is a melanoma.

"Recombinant host cells", "host cells," "cells," "cell cultures," and other such terms denote, for example, microorganisms, insect cells, and mammalian cells, that can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell which has been transformed. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

"Cell line," refers to a population of cells capable of continuous or prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants. The term "cell lines" also includes immortalized cells.

As used herein, the term "microorganism" includes prokaryotic and eukaryotic microbial species such as bacteria and fungi, the latter including yeast and filamentous fungi.

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, particle mediated, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome. Examples of particle mediated transduction are shown in U. S. Patent Nos. 4,945,050 and 5,149,655, which are hereby incorporated by reference in their entireties.

"Purified" and "isolated" mean, when referring to a polypeptide or nucleotide sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. The term "purified" as used herein preferably means at least 75% by weight, more preferably at least 85% by weight, more preferably still at least 95% by weight, and most preferably at least 98% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 1000, can be present).

MAGE-3

MAGE-3 is a tumor antigen recognized on human melanoma cells by autologous cytolytic T lymphocytes. There are approximately 12 closely related genes in the human MAGE gene family, see De Plaen, et al., Immunogenetics, (1994) 40:360-369 and U.S. Pat. No. 5,342,774 which are both hereby incorporated by reference in their entireties. The MAGE proteins are between 309-319 amino acids long. Both the DNA and amino acid sequences are shown in De Plaen et al. and U.S. Pat. No. 5,342,774. MAGE proteins are reported to be expressed in a number of human tumor types, but not to be expressed in normal human tissues, except in testis and placenta. Since MAGE-3 is not significantly expressed in normal tissue, it is a candidate antigen to generate a specific anti tumor T-lymphocyte response. It is the aim of the present invention that a baboon

MAGE-3 homolog be used as a cancer immunotherapeutic agent to generate an immune response against cells expressing the MAGE-3 tumor antigen.

To use a baboon MAGE-3 homolog as a cancer vaccine for humans, it will be preferable to identify those individuals that contain tumors which express MAGE-3. Many techniques may be used to make this diagnosis, for example, reverse transcription and PCR amplification of the RNA of a tumor sample to identify the presence of MAGE-3 mRNA sequences (see Sambrook, et al., MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989), chapter 14 or Gaugler et al., J. Exp. Med (1994) 179:921-930). Also, immunohistochemical techniques or ELISA assays may be used to identify MAGE-3 expressing tumors. Branched DNA testing may be performed as shown in U.S. Patent Nos. 5,124,246, and 4,868,105. After identification, baboon MAGE-3 may be administered to affected individuals by appropriate methods described below.

Baboon MAGE-3 homologs can be produced by a prokaryotic microorganism or an eukaryotic cell that has been transformed with a native or modified baboon MAGE-3 homolog nucleic acid sequence. The baboon MAGE-3 homolog nucleic acid sequence useful in the present invention encodes a protein having an amino acid sequence that is substantially identical to the amino acid sequence of native baboon MAGE-3. Preferably, the baboon MAGE-3 homolog sequence will be homologous to the partial sequences listed in Table 2 below. Preferably, the above sequence will be over 95% homologous to the protein sequence shown in Table 2, more preferably it will be more than 98 % homologous, most preferably more than 99% homologous. Substantial identity of amino acid sequences means the sequences are identical or differ by one or more amino acid alterations (deletion, additions, substitutions) that do not adversely affect the immunogenic utility of the protein.

The precise chemical structure of the baboon MAGE-3 homolog depends on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular protein may be obtained as a acidic or basic salt, or in neutral form. All such preparations which retain their immunogenicity when placed in suitable environmental conditions are included in the definition of proteins herein. Further, the primary amino acid sequence of the protein may be augmented by derivitization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl

groups and the like. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced *in vitro*. In any event, such modifications are included in the definition of protein herein so long as the immunogenicity of the protein is not destroyed. It is expected that such modifications may quantitatively or qualitatively affect the immunogenicity, either by enhancing or diminishing the immunogenicity of the protein, in the various assays. Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or derivatization, and the protein may be cleaved to obtain fragments which retain immunogenicity. Such alterations which do not destroy immunogenicity do not remove the protein sequence from the definition of the baboon MAGE-3 homolog herein.

Finally, modifications to the primary structure itself, by deletion, addition, or alteration of the amino acids incorporated into the sequence during translation, can be made without destroying the immunogenicity of the protein. For example, site specific mutagenesis can enable specific changes in the DNA structure to effect a change in the polypeptide structure. See Mark et al. U. S. Pat. No. 4,959,314, and Sambrook, et al., MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989), Volume 2, chapter 15 which is hereby incorporated by reference in its entirety.

Cross Priming

As discussed above, it is the intent of the present invention to induce an immune response to a self antigen, namely MAGE-3. If a MAGE-3 expressing tumor is growing in a human rather than regressing, it is likely that a state of tolerance or immunosuppression exists in that individual with regard to the MAGE-3 antigen. To overcome this problem, and to increase the level of immune response to this tumor antigen, the present invention utilizes a homolog of MAGE-3 from a different, but related species, such as a baboon. The baboon MAGE-3 homolog molecule will be different enough to be recognized as a foreign antigen more readily than human MAGE-3, but similar enough to enable the immune system to mount a response against the homolog that will also recognize human MAGE-3. This idea is described as "cross priming" and applies to the use of a foreign homolog as an immunogen to elicit an immune response against a self protein.

Without wishing to be bound by theory, it is believed that the baboon MAGE -3 homolog will be a better immunogen because the immune system is more likely to recognize it as foreign and to mount an immune response against it. For example, since human MAGE-3 is a human self antigen, recognition of its T cell and antibody epitopes in a human host will be hindered by self tolerance. Immunization with baboon MAGE-3 homolog(s) may be able to break self tolerance to human MAGE-3 by a process referred to as cross priming or epitope spreading. Baboon MAGE-3 is expected to contain multiple T cell and antibody epitopes similar or identical to human MAGE-3 epitopes ("homologous epitopes") as well as multiple T cell and antibody epitopes that diverge significantly from their human counterparts ("nonhomologous epitopes"). In a human host, the latter epitopes will be seen as foreign and are likely to elicit vigorous immune responses, since no self tolerance will exist.

While not all details of cross priming are completely understood, a central occurrence in the process is the simultaneous presentation of homologous and nonhomologous epitopes by antigen presenting cells (APCs) after immunization of a host with a foreign antigen homologous to a self antigen. Antigen presenting cells include B cells, which are particularly efficient at presenting exogenous antigens that bind specifically to their cell surface antibodies, and other cells such as dendritic cells, Langerhans cells and macrophages that take up antigens by a variety of processes.

In the case of B cells, a self antigen is unlikely to be recognized by surface antibody, taken up and presented due to self tolerance. A foreign homolog of a self antigen is more likely to be taken up by B cell clones that recognize nonhomologous antibody epitopes on the foreign homolog. When this occurs, the entire antigen is processed, including homologous and nonhomologous epitopes, and both classes of epitopes are presented on HLA molecules on the B cell surface. Immunization with the foreign homolog thus results in a greater degree of presentation of homologous epitopes than immunization with the self antigen.

In the case of dendritic cells and macrophages, antigen uptake does not depend on cell surface antibody, but may be enhanced if antigen is complexed with soluble host antibodies and subsequently binds to Fc receptors on the dendritic cells and macrophages. Once again, host antibodies are more likely to recognize the foreign

homolog through its nonhomologous epitopes, resulting in greater uptake and presentation of the foreign homolog than the self antigen.

A further part of the process of cross priming occurs when epitopes of the foreign homolog have been presented on HLA molecules on the surface of either B cells or dendritic cells or macrophages. Helper T cell clones can recognize specific epitopes presented on specific HLA class II molecules, and upon such recognition, deliver signals (via secreted cytokines or up-regulation of surface ligands) that stimulate ("help") the development of antibody-producing B cells and cytolytic T cells. Signals from helper T cell clones can also stimulate antigen presenting cells, resulting in up-regulation of "second signal" ligands on the APC surface. The signals involved in T cell help are of a local nature, and have bystander effects on other T cells, B cells and APCs that are interacting in the local environment.

When a foreign homolog is presented on an APC, the nonhomologous epitopes presented on HLA class II are more likely to be recognized by helper T cell clones than the homologous epitopes shared with the self antigen. Thus, presentation of the foreign homolog will stimulate more helper T cell clones. However, the foreign homolog also contains homologous epitopes, which are presented by the same APCs that are interacting with helper T clones via nonhomologous epitopes. Because of the local nature of T cell help, B cell and T cell clones that recognize homologous epitopes will be stimulated by the help elicited by nonhomologous epitopes, and this bystander stimulation will tend to result in the breaking of tolerance against homologous epitopes.

A baboon sequence containing the nine amino acid sequence EVDPIGHLY is a human HLA-A1 CTL epitope of human MAGE-3. Without wishing to be bound by theory, it is likely that the entire baboon MAGE-3 homolog sequence will encode multiple epitopes similar or identical, to those found in human MAGE- 3, and capable of functional interaction with the products of human HLA alleles. It is also likely that the baboon MAGE-3 homologs contain other epitopes that will be foreign in humans. Some of these foreign epitopes may be expected to generate vigorous immune responses, including T helper responses, and these should increase response to the human-identical epitopes conserved within the baboon homologs. Without wishing to be bound by theory, it is believed that antigen presenting cells will simultaneously present foreign and human-

identical epitopes of baboon MAGE-3 on HLA class I and class II. Helper T cells that recognize the foreign class II epitopes will never have been depleted or anergized, and will be vigorously activated by presentation of the foreign epitopes. The activated helper T cells will in turn release cytokines or express surface ligands that will (1) more fully stimulate the antigen presenting cells; and (2) provide co-stimulation to helper T cells and CTLs that recognize the human-identical epitopes of baboon MAGE-3.

Production

As mentioned previously, recombinant baboon MAGE-3 homologs can be produced by prokaryotic microorganisms or eukaryotic cells. Preferred cell systems include *E. coli*, mammalian, baculovirus, and yeast cells. Preferably, the baboon MAGE-3 homolog is produced by transforming a prokaryotic microorganism with DNA to produce a protein that possesses native baboon MAGE-3 antigenicity. Bacteria are prokaryotic microorganisms that may produce baboon MAGE-3 and *E. coli* is especially preferred. Synthetic recombinant baboon MAGE-3 can also be made in eukaryotes, such as yeast or human cells. See Sambrook, et al., MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989), Volume 3, for bacterial expression-see chapter 17, for expression in mammalian cells-see chapter 16 which are hereby incorporated by reference.

Baboon MAGE-3 homolog DNA can be incorporated into a bacterial expression vector which contains all the control sequences necessary for expressing baboon MAGE-3 polypeptides. Control sequences are known in the art and include: a ribosome binding site, a regulated promoter (i.e. trp, trp-lac, λ p_L, and T7); optionally an operator sequence, an initiation (ATG) and stop codon; an enhancer, etc. It is also preferable to include a origin of replication to facilitate replication of the plasmid within the bacteria.

Appropriate vectors and plasmids are publicly available and can be employed to contain the baboon MAGE-3 homolog DNA. It can be ligated, in operable linkage, to the above control sequences and inserted into the vector using commonly available ligation enzymes and techniques. Additionally, the baboon MAGE-3 homolog DNA sequence can be inserted downstream of a sequence that provides for secretion into the periplasmic space, such as the *pho*A sequence.

A variety of bacterial hosts for expression are known in the art and available from the American Type Culture Collection (ATCC). Bacterial hosts suitable for expressing baboon MAGE-3 include, without limitation: *Campylobacter*, *Bacillus*, *Escherichia*, *Lactobacillus*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*. A typical transformed microorganism useful in the present invention is *E. coli* K-12, strain MM294 (deposited with the American Type Culture Collection on August 4, 1983, by Cetus Corporation under the provisions of the Budapest Treaty and assigned Accession No. 39,405).

Methods of introducing the baboon MAGE-3 homolog DNA into bacterial hosts are well-known in the art, and typically include either treating the bacteria with $CaCl_2$ or other agents, such as divalent cations and DMSO. Naked or plasmid DNA can also be introduced into bacterial cells by electroporation or viral infection. Transformation procedures usually vary with the bacterial species to be transformed. See *e.g.*, (Masson *et al.* (1989) FEMS Microbiol. Lett. 60:273; Palva *et al.* (1982) Proc. Natl. Acad. Sci. USA 79:5582; EP Publ. Nos. 036 259 and 063 953; PCT WO 84/04541, *Bacillus*), (Miller *et al.* (1988) Proc. Natl. Acad. Sci. 85:856; Wang *et al.* (1990) J. Bacteriol. 172:949, *Campylobacter*), (Cohen *et al.* (1973) Proc. Natl. Acad. Sci. 69:2110; Dower *et al.* (1988) Nucleic Acids Res. 16:6127; Kushner (1978) "An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids in Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering (eds. H.W. Boyer and S. Nicosia); Mandel *et al.* (1970) J. Mol. Biol. 53:159; Taketo (1988) Biochem. Biophys. Acta 949:318; Escherichia), (Chassy *et al.* (1987) FEMS Microbiol. Lett. 44:173 *Lactobacillus*); (Fiedler *et al.* (1988) Anal. Biochem 170:38, *Pseudomonas*); (Augustin *et al.* (1990) FEMS Microbiol. Lett. 66:203, *Staphylococcus*), (Barany *et al.* (1980) J. Bacteriol. 144:698; Harlander (1987) "Transformation of *Streptococcus lactis* by electroporation," in Streptococcal Genetics (ed. J. Ferretti and R. Curtiss III); Perry *et al.* (1981) Infect. Immun. 32:1295; Powell *et al.* (1988) Appl. Environ. Microbiol. 54:655; Somkuti *et al.* (1987) Proc. 4th Evr. Cong. Biotechnology 1:412, *Streptococcus*).

Exemplary processes for growing, harvesting, disrupting, or extracting the baboon MAGE-3 polypeptide from cells are substantially described in U.S. Patent Nos. 4,604,377, 4,738,927, 4,656,132, 4,569,790, 4,748,234, 4,530,787, 4,572,298, 5,248,769, and 5,162,507, which are hereby incorporated by reference in their entireties. Preferably, the

MAGE molecules are produced as non-secreted, soluble proteins in *E. coli* and are recovered by 30% ammonium sulfate precipitation after lysing the cells in a microfluidizer.

Baboon MAGE-3 homologs can be expressed in a variety of other expression systems; for example, preferably mammalian or baculovirus expression systems, as well as yeast systems.

Mammalian expression systems are known in the art. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element, usually located within 100 to 200 bp upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation, Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd ed. 1989).

The presence of an enhancer element (enhancer), combined with the promoter elements described above, will usually increase expression levels. An enhancer is a regulatory DNA sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are also active when they are placed upstream or downstream from the transcription initiation site, in either normal or flipped orientation, or at a distance of more than 1000 nucleotides from the promoter, Maniatis et al., Science 236:1237 (1989); Alberts et al. Molecular Biology of the Cell, 2nd ed (1989). Enhancer elements derived from viruses may be particularly useful, because they usually have a broader host range. Examples include the SV40 early gene enhancer, Dijkema et al (1985) EMBO J. 4:761, and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, Gorman et al. (1982) Proc. Natl. Acad. Sci. 79:6777, and from human cytomegalovirus, Boshart et al. (1985) Cell 41:5221. Additionally, some enhancers are regulatable and become active only in the presence of an inducer, such as a hormone or

metal ion, Sassone-Corsi et al. (1986) Trends Genet. 2:215; Maniatis et al. (1987) Science 236:1237.

A protein molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, the N-terminal methionine may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Alternatively, foreign proteins can also be secreted from the cell into the growth medium by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

Usually, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation, Birnstiel et al. (1985) Cell 41:349; Proudfoot and Whitelaw (1988) "Termination and 3' end processing of eukaryotic RNA. In Transcription and splicing (ed. B.D. Hames and D.M. Glover); Proudfoot (1989) Trends Biochem. Sci. 14:105. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator/polyadenylation signals include those derived from SV40, Sambrook et al (1989), Molecular Cloning: A Laboratory Manual.

Some genes may be expressed more efficiently when introns are present. Several cDNAs, however, have been efficiently expressed from vectors that lack splicing signals (also called splice donor and acceptor sites), see e.g., Gething and Sambrook (1981)

Nature 293:620. Introns are intervening noncoding sequences within a coding sequence that contain splice donor and acceptor sites. They are removed by splicing following polyadenylation of the primary transcript, Nevins (1983) Annu. Rev. Biochem. 52:441; Green (1986) Annu. Rev. Genet. 20:671; Padgett et al. (1986) Annu. Rev. Biochem. 55:1119; Krainer and Maniatis (1988) "RNA splicing," In Transcription and splicing (ed. B.D. Hames and D.M. Glover).

Usually, the above-described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be included in an expression construct, if desired. Mammalian replication systems include those derived from animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40, Gluzman (1981) Cell 23:175, or polyomavirus, replicate to extremely high copy number in the presence of the appropriate viral T antigen. Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the replicon may have two replication systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a procaryotic host for cloning and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2, Kaufman et al. (1989) Mol. Cell. Biol. 9:946, and pHEBO, Shimizu et al. (1986) Mol. Cell. Biol. 6:1074.

The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), and a number of other cell lines.

The baboon MAGE-3 homolog nucleic acid sequence can also be inserted into a suitable insect expression vector, and is operably linked to the control elements within that vector. Vector construction employs techniques which are known in the art.

Generally, the components of the expression system include a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the heterologous gene or genes to be expressed; a wild type baculovirus with a sequence homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous recombination of the heterologous gene into the baculovirus genome); and appropriate insect host cells and growth medium.

After inserting the DNA sequence encoding the protein into the transfer vector, the vector and the wild type viral genome are transfected into an insect host cell where the vector and viral genome are allowed to recombine. The packaged recombinant virus is expressed and recombinant plaques are identified and purified. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, inter alia, Invitrogen, San Diego CA ("MaxBac" kit). These techniques are generally known to those skilled in the art and fully described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987) (hereinafter "Summers and Smith").

Prior to inserting the DNA sequence encoding the protein into the baculovirus genome, the above-described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are usually assembled into an intermediate construct (transfer vector).

Currently, the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, have also been designed. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 base pairs downstream from the ATT; see Luckow and Summers, Virology (1989) 17:31.

The plasmid usually also contains the polyhedron polyadenylation signal (Miller et al. (1988) Ann. Rev. Microbiol., 42:177) and a procaryotic ampicillin-resistance (amp) gene and origin of replication for selection and propagation in E. coli.

Baculovirus transfer vectors usually contain a baculovirus promoter. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A baculovirus transfer vector may also have a second domain called an enhancer, which, if present, is usually distal to the structural gene. Expression may be either regulated or constitutive.

Structural genes, abundantly transcribed at late times in a viral infection cycle, provide particularly useful promoter sequences. Examples include sequences derived from the gene encoding the viral polyhedron protein, Friesen et al., (1986) "The Regulation of Baculovirus Gene Expression," in: The Molecular Biology of Baculoviruses (ed. Walter Doerfler); EPO Publ. Nos. 127 839 and 155 476; and the gene encoding the p10 protein, Vlak et al., (1988), J. Gen. Virol. 69:765.

DNA encoding suitable signal sequences can be derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene (Carbonell et al. (1988) Gene, 73:409). Alternatively, since the signals for mammalian cell posttranslational modifications (such as signal peptide cleavage, proteolytic cleavage, and phosphorylation) appear to be recognized by insect cells, and the signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate cells and vertebrate cells, leaders of non-insect origin, such as those derived from genes encoding human α-interferon, Maeda et al., (1985), Nature 315:592; human gastrin-releasing peptide, Lebacq-Verheyden et al., (1988), Molec. Cell. Biol. 8:3129; human IL-2, Smith et al., (1985) Proc. Nat'l Acad. Sci. USA, 82:8404; mouse IL-3, (Miyajima et al., (1987) Gene 58:273; and human glucocerebrosidase, Martin et al. (1988) DNA 7:99, can also be used to provide for secretion in insects.

After insertion of the DNA sequence and/or the gene encoding the expression product precursor of the protein, an insect cell host is co-transformed with the heterologous DNA of the transfer vector and the genomic DNA of wild type baculovirus -- usually by co-transfection. Methods for introducing heterologous DNA into the desired site in the baculovirus virus are known in the art. (See Summers and Smith; Ju et al. (1987); Smith et al., Mol. Cell. Biol. (1983) 3:2156; and Luckow and Summers (1989)). For example, the insertion can be into a gene such as the polyhedrin gene, by

homologous double crossover recombination; insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene. Miller et al., (1989), Bioessays 4:91.

The newly formed baculovirus expression vector is subsequently packaged into an infectious recombinant baculovirus. Methods to identify recombinant viruses are described in "Current Protocols in Microbiology" Vol. 2 (Ausubel et al. eds) at 16.8 (Supp. 10, 1990); Summers and Smith; Miller et al. (1989).

Recombinant baculovirus expression vectors have been developed for infection into several insect cells. For example, recombinant baculoviruses have been developed for, inter alia: *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni* (PCT Pub. No. WO 89/046699; Carbonell et al., (1985) J. Virol. 56:153; Wright (1986) Nature 321:718; Smith et al., (1983) Mol. Cell. Biol. 3:2156; and see generally, Fraser, et al. (1989) In Vitro Cell. Dev. Biol. 25:225).

Yeast expression systems are also known to one of ordinary skill in the art. Although less preferred in the present invention, such systems may be used. For a general review of yeast expression, see Barr et al. (eds.), Yeast Genetic Engineering, Butterworths, London (1989).

A baboon MAGE-3 homolog protein may be purified after expression in a host cell system by a sequence of recovery and purification steps. For instance, baboon MAGE-3 expressed as a soluble protein in *E. coli* may be released by breaking the bacterial cells in a microfluidizer and recovered by ammonium sulfate precipitation. MAGE-3 protein may then be redissolved in buffer and purified by a variety of steps including, for example, anion exchange chromatography, size exclusion chromatography, hydroxyapatite chromatography, hydrophobic interaction chromatography, metal chelation chromatography, reverse phase HPLC, affinity chromatography, and further ammonium sulfate precipitations. These techniques are well known to those of skill in the art.

Formulation and Administration

After the baboon MAGE-3 homolog is produced and purified it may be incorporated into a pharmaceutical formulation for application as a single or combination antigen in human and veterinary therapeutics therapy, such as cancer therapy. As a formulation, it is administered to the subject by methods known in the art (see below). This formulation may contain other compounds that increase the effectiveness or promote the desirable qualities of the baboon MAGE-3 homolog. The composition must be safe for administration via the route that is chosen, it must be sterile, retain immunogenicity, and it must stably solubilize or suspend the baboon MAGE-3 homolog. To maintain the sterility and to increase the stability of the baboon MAGE-3 homolog, the composition may be lyophilized and reconstituted prior to use. These formulations may either be prophylactic (to prevent cancer) or therapeutic (to treat established cancer).

Such vaccines comprise an antigen or antigens, usually in combination with "pharmaceutically acceptable carriers", which include any carrier that does not itself induce the production of immune responses harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid, such as a toxoid from diphtheria, tetanus, cholera, *H. pylori*, etc. pathogens. See also, U. S. Patent No. 4,877,611 which is hereby incorporated by reference in its entirety.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (PCT Publ. No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5%

pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphoryl lipid A (MPL) (see U. S. Patent No. 4,877,611), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant); (5) cytokines, such as interleukins (IL-1, IL-2, IL-12 etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; and (6) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59 are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-iso-glutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

The immunogenic formulations (e.g., the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Sugars or sugar alcohols can be included in the baboon MAGE-3 compositions. Sugar is generally defined as mono, di, or polysaccharides, or water-soluble glucans, including for example, fructose, glucose, mannose, sorbose, xylose, maltose, lactose, sucrose, dextran, pullulan, dextrin, cyclodextrin, soluble starch, hydroxyethyl starch, and carboxymethylcellulose-Na. Sucrose is the most preferred. Sugar alcohol is defined as the corresponding alcohols of the above sugars, and includes for example mannitol, sorbitol, inositol, galactitol, dulcitol, xylitol, and arabitol; mannitol is the most preferred. The sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to the amount used as long as the sugar alcohol is soluble in the

aqueous preparation. Preferably, the sugar or sugar alcohol concentration is between 1.0 w/v% and 7.0 w/v%, more preferably between 2.0 and 6.0 w/v %.

It is also preferred to use a buffer in the composition to minimize pH changes in the solution before lyophilization or after reconstitution. Almost any physiological buffer may be used, but citrate, phosphate, succinate, or glutarate buffers, or mixtures thereof are preferred. Most preferred is a citrate buffer. Preferably, the concentration is from 0.01 to 0.3 M.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be present in controlled release formulations and emulsified or encapsulated in liposomes for enhanced adjuvant effect, as discussed above under pharmaceutically acceptable carriers. For example, there is a great deal of literature on liposomes that are useful to deliver proteins. The following U.S. patents are hereby incorporated by reference in their entireties: 4,863,740, 4,877,561, 5,225,212, 5,007,057, 5,049,389, 5,023,087, 4,992,271, 4,962,091, 4,895,719, 4,855,090, 4,844,904, 4,781,871, 4,762,720, 4,752,425, 4,612,007, 5,292,524, 5,258,499, 5,229,109, 4,983,397, 4,895,719, and 4,684,521. Additionally, multivesicullar vesicles and microcapsules are also envisioned by the present invention, see WO 94/23697 and U.S. Patent No. 5,102,872 respectively. Baboon MAGE-3 may be entrapped in or conjugated to polymers and/or implanted in a patient to facilitate slow release. Examples of these technologies are shown in U.S. Patent Nos. 5,110,596, 5,034,229, 4,766,106 and 5,057,318 which are hereby incorporated by reference in their entireties. Alternatively, other baboon MAGE-3 homolog formulations resulting in equivalent immune stimulation for one day to two weeks could be utilized.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount," it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g., human, nonhuman primate, etc.), the capacity of the

individual's immune system to synthesize antibodies and to generate specific T cell responses, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The baboon MAGE-3 homolog may be injected parenterally, intravenously, intraperitoneally, intraarterially, subcutaneously, or intradermally. Preferably, the route of administration is intramuscular, subcutaneous, or intradermal. Preferably, the maximum dose for humans is 1000 μg, more preferably it is 100 μg, most preferably 75 μg,. Preferably, the minimum human dose is 0.1 μg, more preferably the minimum human dose is 10 μg, most preferably the minimum human dose is 25 μg.

Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. Preferably, the baboon MAGE-3 homolog is administered once per week. The vaccine may be administered in conjunction with other immunoregulatory agents.

The present baboon MAGE-3 homologs may be administered along with other antigens, monoclonal antibodies, or immunotoxins. The antigens or antibodies may be directed to other melanoma proteins, See U. S. Pat. Nos. 5,006,470, 5,005,559, 4,877,611, 4,590,071, 4,851,510 and 4,863, 854 which are hereby incorporated by reference in their entireties.

DNA Immunization

The baboon MAGE-3 homolog nucleic acid sequence can be used to treat humans via gene therapy. Strategies for delivery of the baboon MAGE-3 nucleic acid sequence will utilize viral and non viral vector approaches and *in vivo* and *ex vivo* gene transfer (see U. S. Patent No. 5,399,346 for *ex vivo* therapy). Gene expression can be induced using endogenous mammalian or heterologous promoters. Expression of the gene *in vivo* is preferably regulated. Regulation is shown below.

For viral gene therapy, the baboon MAGE-3 homolog nucleic acid sequence will be inserted into retroviral vectors, adenoviral vectors, adeno associated viral vectors and

sindbis vectors (for retroviral vectors, see Kimura, et al. Human Gene Therapy, 6:185-193 (1995): for adeno associated viral vectors, see Kaplitt et al., Nature Genetics 8(2):148-153 (1994). Promoters used in these examples include the Moloney retroviral LTR, CMV promoter and the mouse albumin promoter. Replication competent free virus will be produced and can be injected directly into the animal or by transduction of autologous cells *ex vivo* then injected *in vivo* (see Zatloukal et al., PNAS 91:5148-4152 (1994). See also Anderson et al., U. S. Pat. No. 5,399,346 which is hereby incorporated by reference in its entirety.

Also, the baboon MAGE-3 homolog nucleic acid sequence can be used by itself or it can be inserted into a plasmid and used for expression *in vivo* or *ex vivo*. For *in vivo* therapy, DNA will be delivered by direct injection into tissue or by intravenous infusion (for particle mediated transfer of genes, see U. S. Patent Nos. 5,149,655, 5,120,567, and 4,945,050, for the use of naked DNA see WO 93/19183, all of which are hereby incorporated by reference in their entireties). Promoters include endogenous and heterologous promoters. DNA will be injected in formulation buffers that will stabilize the DNA, facilitate the transduction into cells, and/or provide targeting (Zhu et al, Science, 261:209-211 (1993).

Regulation of the baboon MAGE-3 homolog nucleic acid sequence expression *in vivo* by either viral or non-viral vector systems can be regulated for maximal efficacy and safety by use of regulated gene expression promoters (Gossen et al., PNAS, 89:5547-5551 (1992). The baboon MAGE-3 homolog gene can be regulated by tetracycline responsive promoters, or in positive or negative systems.

The gene therapy methods of the present invention may be specifically controlled by methods for controlling gene expression *in vivo*. For example, genes may be introduced into cells under the control of promoters which are activated using radiotherapy. For example, U.S. Patent No. 5,205,152 entitled "Cloning and Expression of Early Growth Regulatory Protein Genes" shows that the Egr-1 gene is one of the best radiation induced genes and may be activated by exposure to radiation. WO 92/11033 disclosed genetic constructs which comprise an enhancer-promoter region which is responsive to radiation, and at least one structural gene whose expression is controlled by the enhancer-promoter. In this embodiment the baboon MAGE-3 homolog nucleic acid sequence may be under the

control of the specific enhancer-promoter region. The U.S. Patent and the PCT application are hereby incorporated by reference in their entireties.

Examples

Total RNA was isolated from 1.8 grams of frozen baboon testis by a guanidine thiocyanate tissue disruption method (Promega Protocols and Applications Guide, 1991, pg. 125-128). This RNA was used as the target for RT-PCR with M-MLV reverse transcriptase, Taq DNA Polymerase (both from Promega), and random hexamers as primer for second strand synthesis (as described by E.S. Kawasaki in PCR Protocols: A Guide to Methods and Applications, 1990, pg. 21-27). PCR primers were designed to amplify a region of human MAGE-3 containing two immunologically important T-cell epitopes (De Plaen et al., Immunogenetics (1994) 40: 360-369). Two forward primers and two reverse primers were used. Each forward and each reverse primer were made to anneal to regions near each other. One forward primer did not produce fragments with either reverse primer, but the other forward primer produced a PCR product of about 400 bp, with each reverse primer. The forward primer used successfully was CCTTGCAGCTGGTCTTTGGC and two reverse primers were GCAGGACTTTCACATAGCTGG and GGGTAGGAAATGTGAGGTCCTC.

Both primers contained extensions that inserted restriction sites. The forward primers incorporated a 5' Kpn I restriction site, while the reverse primers incorporated a 5' Pst I site. Each PCR product was purified in an agarose gel with the Geneclean system, digested with Kpn I + Pst I, and then re-purified in a similar fashion. The plasmid pUC19 was likewise digested and purified. The MAGE-3-like fragments were ligated into pUC19 and used to transform competent *E. coli* DH5a bacteria by standard methods (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., 1989). Plasmid mini-preps were made from overnight cultures grown from single colonies (Promega Wizard Mini-Prep columns). Plasmid DNAs were digested with Kpn I + Pst I (Promega), and those which released fragments of the expected size were used for DNA sequencing (Sanger method using Sequencing Grade Taq Polymerase from Promega). The results are shown in Table 1 for the DNA sequences and Table 2 for the expected amino acid sequences.

Table 1. DNA sequence alignments of five MAGE-3-like mRNAs from baboon testis with human MAGE-3

```
                                        I                   A
D                       A
Hu:TTTGGCATCGAGCTGATGGAAGTGGACCCCATCGGCCACTTGTACATCTTTGCCACCTGCCTGGGCC
TCTCCTACGATGGCCTGCTGGGTGACAATCAGATCATGCCCAAGGCA
                                                    X
T
A
:TTTGGCATCGAGCTGATGGAAGTGGACCCAATTGGCCACTTGTACATCTTTGWCACCTGCCTGGGCCTC
TCCTACGATGGCCTGCTGGGTGACAATCAGATCATGCCCAAGACA
                                        V           X
T
C
:TTTGGCATCGAGCTGATGGAAGTGGACCCAGTCGGCCACTTGTACATCTTTGWCACCTGCCTGGGCCTC
TCCTACGATGGCCTGCTGGGTGACAATCAGATCATGCCCAAGACA
                                        V
N                       T
D
:TTTGGCATCGAGCTGATGGAAGTGGACCCCGTCGGCCACTTGTACATCTTTGCCACCTGCCTGGGCCTC
TCCTACGATGGCCTCCTGGGCAACAATCAGATCATGCCCAAGACA
                                        V
B
:TTTGGCATCGAGCTGATGGAAGTGGACCCAGTCGGCCACTTGTACATCTTTGCCACCTGCCTGGGCCTC
TCCTACGATGGCCTGCTGGGCGACAATCAGATCATGCCCAAGGCA
                                        V
F
:TTTGGCATCGAGCTGATGGAAGTGGACCCAGTCGGCCACTTGTACATCTTTGCCACCTGCCTGGGCCTC
TCCTACGATGGCCTGCTGGGCGACAATCAGATCATGCCCAAGGCA

                    V       I       R               A
E           D
Hu:GGCCTCCTGATAATCGTCCTGGCCATAATCGCAAGAGAGGGCGACTGTGCCCCTGAGGAGAAAATCT
GGGAGGAGCTGAGTGTGTTAGAGGTGTTTGAGGGGAGGGAAGACAGT
```

```
                    L       -       K
H
A :GGCCTTCTGATAATCCTCCTGGCC---
ATCGCAAAAGAGGGCGACTGCGCCCCCGAGGAGAAAATCTGGGAGGAGCTGAGTGTGTTGGAGGTGTTTG
AGGGGAGGGAGCACAGT
                    L       -       K
H
C :GGCCTTCTGATAATCCTCCTGGCC---
ATCGCAAAAGAGGGCGACTGCGCCCCCGAGGAGAAAATCTGGGAGGAGCTGAGTGTGTTGGAGGTGTTTG
AGGGGAGGGAGCACAGT
                        T       K
H
D
:GGCCTCCTGATAATCGTCCTGACCATAATCGCAAAAGAGGGCGACTGCGCCCCTGAGGAGAAAATCTGG
GAGGAGCTGAGTGTGTTGGAGGTGTTTGAGGGGAGGGAGCACAGT
                                    K
D
B
:GGCCTCCTGATAATCGTCCTGGCCATAATCGCAAAAGAGGGCGACTGCGCCCCTGAGGAGAAAATCTGG
GAGGAGCTGAGTGTGTTGGAGGTGTTTGATGGGAGGGAGGACAGT
                                    K           T
D
F
:GGCCTCCTGATAATCGTCCTGGCCATAATCGCAAAAGAGGGCGACTGCACCCCTGAGGAGAAAATCTGG
GAGGAGCTGAGTGTGTTGGAGGTGTTTGATGGGAGGGAGGACAGT


    L G     K               H                       R
V                   C Y E
Hu:ATCTTGGGGGATCCCAAGAAGCTGCTCACCCAACATTTCGTGCAGGAAAACTACCTGGAGTACCGGC
AGGTCCCCGGCAGTGATCCTGCATGTTATGAATTCCTGTGGGGTCCA
    F A     R               D                       W
V                   C Y E
A
:ATCTTCGCGGATCCCAGGAAGCTGCTCACCCAAGATTTTGTGCAGGAAAACTACCTGGAGTACTGGCAG
GTGCCCGGCAGTGATCCTGCATGCTACGAGTTCCTGTGGGGTCCA
    F A     R               D
C :ATCTTCGCGGATCCCAGGAAGCTGCTCACCCAAGATTTTGTGC
```

```
      F  A        R                D
D :ATCTTCGCGGATCCCAGGAAGCTGCTCACCCAAGATTTCGTGC
   X  F  A        R                D                              R
V                    C  Y  E  ?
B
:KTCTTCGCGGATCCCAGGAAGCTGCTCACCCAAGATTTCGTGCAGGAAAACTACCTGGAGTACCGGCAG
GTGCCAGGGAGTGAGCCTGCATGCTACGAGNGCCTGTGGGGTCCA
   X  F  A        R                D
F :KTCTTCGCGGATCCCAGGAAGCTGCTCACCCAAGATTTCGTGC

         L                       V
Hu:AGGGCCCTCGTTGAAACCAGCTATGTGAAAGTCCTGC
         ?                       ?
A :AGGGCCCNCGTTGAAACCAGCTATGTGAAAGNCCTGT
         ?                       ?
B :AGGGCCCNCGTTGAAACCAGCTATGTGAAAGNCCTGT

W = A or T, K = G or T
```

Table 2. Amino acid sequence alignments of five MAGE-3-like mRNAs from baboon testis with human MAGEs

```
      162              171              181              191
      201              211              221              231
      241
Hu:   FGIELMEVD PIGHLYIFAT CLGLSYDGLL GDNQIMPKAG LLIIVLAIIA REGDCAPEEK
IWEELSVLEV FEGREDSILG DPKKLLTQHF
A:    ......... ........B. .......... ........T. ....L..-.. K.........
.......... .....H..FA ..R.....D.
C:    ......... .V......B. .......... ........T. ....L..-.. K.........
.......... .....H..FA ..R.....D.
D:    ......... .V........ .......... .N......T. ......T... K.........
.......... .....H..FA ..R.....D.
B:    ......... .V........ .......... .......... .......... K.........
.......... .D.....JFA ..R.....D.
F:    ......... .V........ .......... .......... .......... K....T....
.......... .D.....JFA ..R.....D.

      251              261              271
Hu:   VQENYLEYRQ VPGSDPACYE FLWGPRALVE TSYVKVL
A:    ........W. .......... .......?.. .....?.
B:    .......... ....E..... ?......?.. .....?.
```

B = V or D

J = V or F

? = amino acid could not be determined due to DNA sequence ambiguity

These gene fragments of baboon MAGE-3 homologs can be used to probe a cDNA library made from poly-A purified baboon testis mRNA. This will allow isolation of full length cDNA clones of baboon MAGE-3 homologs, without concern over point mutations possibly introduced by Taq DNA polymerase during PCR. To test the usefulness of a fragment as a probe, a fragment with DNA sequence B in Table 1 was isolated and radiolabeled with 32P-dCTP (Amersham Rediprime DNA labeling system)

and was successfully used to bind to plasmid DNA containing a baboon MAGE-3 sequence immobilized on a nylon membrane by Southern blotting. Poly-A mRNA has been purified from total baboon testis RNA (mRNA purification kit from Pharmacia). PCR of this preparation with MAGE-3 primers produces product of the expected size, indicating that the mRNA preparation contains baboon MAGE-3 homolog messages. The next step will be to carry out a Northern blot hybridization, again using sequence B fragment as probe. If this Northern hybridization is successful, knowledge of the size of the complete baboon MAGE-3 homolog messages will be useful in the subsequent production and screening of a cDNA library.

Human Use

Human patients who are suffering from a MAGE-3 positive tumor can benefit from vaccination with baboon MAGE-3 homologs. When the tumor is diagnosed as having MAGE-3 positive cells (by a branched DNA test or immunohistochemistry), baboon MAGE-3 will be administered intramuscularly, subcutaneously or intradermally. The baboon MAGE-3 will be administered in combination with the vaccine adjuvant MF59 to achieve enhanced immune responses. The dosage of baboon MAGE-3 will be between 25 and 100 μg once a week for six weeks, and blood samples will be drawn from the patient to monitor MAGE-3 specific antibody and T cell responses. If an insufficient level of response is observed, then other dose levels or schedules will be investigated or MTP-PE will be included in the MF59 adjuvant. If ongoing MAGE-3 specific immune responses or tumor regression are observed, but complete tumor regression has not occurred in six weeks, dosing may be continued over a longer period.

Patients who are at high risk of developing cancer types that tend to express MAGE-3 may be vaccinated as a prophylactic measure to prevent cancer development, using a dose, route and schedule determined to be effective in inducing MAGE-3 specific immune responses.

Claims:

1. An isolated nucleic acid sequence, comprising the nucleic acid sequences A, B, C, D, or F of Table 1, and sequences which hybridize to any of sequences A, B, C, D, or F under stringent conditions.

2. An isolated nucleic acid sequence, selected from the group consisting of the nucleic acid sequences A, B, C, D, or F of Table 1.

3. An isolated nucleic acid sequence of claim 1 comprising sequence A of Table 1.

4. An isolated nucleic acid sequence of claim 1 comprising sequence B of Table 1.

5. An isolated nucleic acid sequence of claim 1 comprising sequence C of Table 1.

6. An isolated nucleic acid sequence of claim 1 comprising sequence D of Table 1.

7. An isolated nucleic acid sequence of claim 1 comprising sequence F of Table 1.

8. An expression vector comprising the nucleic acid sequence of claim 1 operably linked to the appropriate control sequences for expressing the nucleic acid sequence.

9. An expression vector comprising the nucleic acid sequence of claim 2 operably linked to the appropriate control sequences for expressing the nucleic acid sequence.

10. An expression vector comprising the nucleic acid sequence of claim 3 operably linked to the appropriate control sequences for expressing the nucleic acid sequence.

11. An expression vector comprising the nucleic acid sequence of claim 4 operably linked to the appropriate control sequences for expressing the nucleic acid sequence.

12. An expression vector comprising the nucleic acid sequence of claim 5 operably linked to the appropriate control sequences for expressing the nucleic acid sequence.

13. An expression vector comprising the nucleic acid sequence of claim 6 operably linked to the appropriate control sequences for expressing the nucleic acid sequence.

14 An expression vector comprising the nucleic acid sequence of claim 7 operably linked to the appropriate control sequences for expressing the nucleic acid sequence.

15. A host cell containing the expression vector of claim 8.

16. A host cell containing the expression vector of claim 9.

17. A host cell containing the expression vector of claim 10.

18. A host cell containing the expression vector of claim 11.

19. A host cell containing the expression vector of claim 12.

20. A host cell containing the expression vector of claim 13.

21. A host cell containing the expression vector of claim 14.

22. A process for making a baboon MAGE-3 polypeptide comprising expressing the nucleic acid sequence of claim 1.

23. A process for making a baboon MAGE-3 polypeptide comprising expressing the nucleic acid sequence of claim 2.

24. A process for using the nucleic acid sequence of claim 1 comprising introducing the nucleic acid sequence into a human to stimulate an immune response to the encoded polypeptide.

25. A process for using the nucleic acid sequence of claim 2 comprising introducing the nucleic acid sequence into a human to stimulate an immune response to the encoded polypeptide.

26. A process in accordance with claim 24, wherein the nucleic acid sequence is directly administered to the human.

27. A process in accordance with claim 25, wherein the nucleic acid sequence is directly administered to the human.

28. A process in accordance with claim 24, wherein the nucleic acid sequence is introduced into cells and those cells are introduced into the human.

29. A process in accordance with claim 25, wherein the nucleic acid sequence is introduced into cells and those cells are introduced into the human.

30. A polypeptide comprising a baboon MAGE-3 polypeptide selected from the group consisting of sequences A, B, C, D, or F from Table 2.

31. A polypeptide comprising an immunologically active fragment of the baboon MAGE-3 polypeptide shown in sequences A, B, C, D, or F from Table 2

32. A formulation of the polypeptide shown in claim 30 further comprising an adjuvant.

33. A formulation of the polypeptide shown in claim 31 further comprising an adjuvant.

34. A formulation of the polypeptide shown in claim 32, wherein the adjuvant is selected from the group consisting of MF 59, MF-59 plus MTP-PE, and alum.

35. A formulation of the polypeptide shown in claim 33, wherein the adjuvant is selected from the group consisting of MF 59, MF-59 plus MTP-PE, and alum.

36. An immunostimulatory method comprising administering a baboon MAGE-3 polypeptide to a human to stimulate the immune system of a human.

37. An immunostimulatory method in accordance with claim 36, wherein the MAGE-3 polypeptide has a sequence substantially shown in sequences A, B, C, D, or F from Table 2.

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(57) Abstract

MAGE-3 tumor antigens are useful for overcoming tolerance to tumors.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N5/10 A61K48/00 C07K14/705 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 23031 A (LUDWIG INST CANCER RES) 13 October 1994 see SEQ ID NO:11 nucleotides 654-1033, and page 48 line 24 to page 50. ---	1-21, 24-29, 32-35
X	JOURNAL OF EXPERIMENTAL MEDICINE, vol. 179, no. 3, 1 March 1994, pages 921-930, XP000614538 B. GAUGLER ET AL.: "HUMAN GENE MAGE-3 CODES FOR AN ANTIGEN RECOGNIZED ON A MELANOMA BY AUTOLOGOUS CYTOLYTIC T LYMPHOCYTES" see nucleotides 640-1014 of the MAGE-3 sequence in figure 5 and page 925 last paragraph to page 928 --- -/--	1-21, 24-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

Inter onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOGENETICS, vol. 40, no. 5, 1994, pages 360-369, XP000614537 PLAEN DE E ET AL: "STRUCTURE, CHROMOSOMAL LOCALIZATION, AND EXPRESSION OF 12 GENES OF THE MAGE FAMILY" cited in the application see figure 2 nucleotides 349-927 of the MAGE-3 sequence ---	1-21, 24-29
A	GENOMICS, vol. 28, no. 1, 1 July 1995, pages 74-83, XP000670311 O. DE BACKER ET AL.: "STRUCTURE, CHROMOSOME LOCATION, AND EXPRESSION PATTERN OF THREE MOUSE GENES HOMOLOGOUS TO THE HUMAN MAGE GENES" see the whole document -----	1-37

INTERNATIONAL SEARCH REPORT

ernational application No.

PCT/US 96/16319

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 24-29,36,37
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although these claims are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

* see continuation-sheet *

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

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Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/ 16319

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Claims 1-7 refer to nucleic acid sequences A,B,C,D or F of Table 1. However, even after careful inspection of Table 1 it was not possible to determine which sequences were meant. Nonetheless, in combination with Table 2, which at least gives the amino acid sequences A,B,C,D and F, the following table of equivalency could be made:

SEQ.ID.NO:2 = nucleic acid sequence A
SEQ.ID.NO:5 = nucleic acid sequence B
SEQ.ID.NO:3 = nucleic acid sequence C
SEQ.ID.NO:4 = nucleic acid sequence D
SEQ.ID.NO:6 = nucleic acid sequence F

In order tobe clear within the meaning of Article 6 PCT, claims 1-7 should refer to the respective SEQ.ID. numbers.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9423031 A	13-10-94	AU 668772 B	16-05-96
		AU 5096993 A	29-03-94
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